

AR226-0089

PFOS:
A 96-HOUR SHELL DEPOSITION TEST
WITH THE EASTERN OYSTER (*Crassostrea virginica*)

FINAL REPORT

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454A-106

3M LAB REQUEST NO. U2723

U. S Environmental Protection Agency
Series 850 – Ecological Effects Test Guidelines
OPPTS Number 850.1025

AUTHORS:

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STUDY INITIATION DATE: May 3, 1999

STUDY COMPLETION DATE: August 11, 1999

AMENDED REPORT DATE: April 26, 2000

Submitted to

3M Corporation
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St. Paul, Minnesota 55144

Wildlife International Ltd.

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Easton, Maryland 21601
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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: 3M Corporation

TITLE: PFOS: A 96-Hour Shell Deposition Test with the Eastern Oyster (*Crassostrea virginica*)

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454A-106

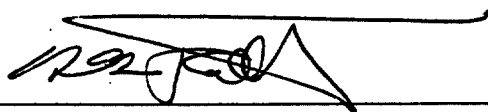
STUDY COMPLETION: August 11, 1999

AMENDED REPORT: April 26, 2000

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice, OCDE/GD (92) 32, Environment Monograph No. 45, Paris 1992; and Japan MAFF, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984 with the following exceptions:

The test substance was not characterized in accordance with full GLP compliance; however, the characterization was performed according to 3M Standard Operating Procedures and Methods, and all raw data are being maintained in the 3M archives. The test substance is being recharacterized in accordance with GLP.

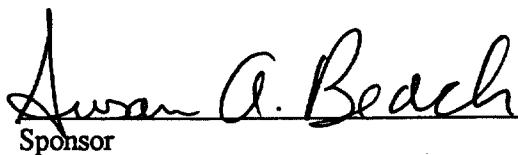
The stability of the test substance under conditions of storage at the test site was not determined in accordance with Good Laboratory Practice Standards.

STUDY DIRECTOR:

Kurt R. Drott
Senior Biologist

4/26/00

DATE

SPONSOR APPROVAL:
Sponsor

4/27/00

DATE

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QUALITY ASSURANCE STATEMENT

This study was examined for compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice, OCDE/GD (92) 32, Environment Monograph No. 45, Paris 1992; and Japan MAFF, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984. The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Laboratory Management were as follows:

ACTIVITY:	DATE CONDUCTED:	DATE REPORTED TO:	
		STUDY DIRECTOR:	MANAGEMENT:
Test Substance Preparation	May 21, 1999	May 21, 1999	May 21, 1999
Test Initiation and Matrix Fortification Preparation	May 24, 1999	May 24, 1999	May 26, 1999
Biological Data and Draft Report	July 1, 1999	July 1, 1999	July 2, 1999
Analytical Data and Draft Report	July 1, 1999	July 1, 1999	July 2, 1999
Final Report	August 11, 1999	August 11, 1999	August 11, 1999
Amended Report	April 25, 2000	April 25, 2000	April 26, 2000

James H. Coleman
James H. Coleman
Quality Assurance Representative

4-26-00
DATE

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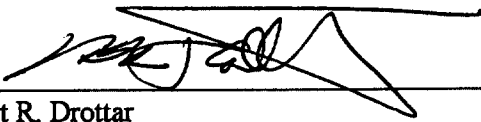
REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFOS: A 96-Hour Shell Deposition Test with the Eastern Oyster (*Crassostrea virginica*)

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STUDY DIRECTOR:

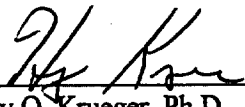


Kurt R. Drott
Senior Biologist

4/26/00

DATE

MANAGEMENT:



Henry O. Krueger, Ph.D.
Director, Aquatic Toxicology and
Non-Target Plants

4/26/00

DATE

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SUMMARY

SPONSOR:	3M Corporation
SPONSOR'S REPRESENTATIVE:	Ms. Susan A. Beach
LOCATION OF STUDY, RAW DATA AND A COPY OF THE FINAL REPORT:	Wildlife International Ltd. Easton, Maryland 21601

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER:	454A-106
TEST SUBSTANCE:	PFOS (Perfluorooctane Sulfonic Acid Potassium Salt)
STUDY:	PFOS: A 96-Hour Shell Deposition Test with the Eastern Oyster (<i>Crassostrea virginica</i>)
MEAN MEASURED TEST CONCENTRATIONS:	Negative Control, 0.36, 0.40, 1.3, 1.9 and 3.0 mg a.i./L
TEST DATES:	Experimental Start – May 24, 1999 Biological Termination – May 28, 1999 Experimental Termination – June 14, 1999
LENGTH OF TEST:	96 Hours

TEST ORGANISM:	Eastern Oyster (<i>Crassostrea virginica</i>)
SOURCE OF TEST ORGANISMS:	P. Cummins Oyster Company, Inc. 618 West 33 rd Street Baltimore, Maryland 21211
OYSTER LENGTH (N = 20):	Mean = 33.8 mm, Range = 27.8 to 41.5 mm

96-HOUR EC50:	>3.0 mg a.i./L
95% CONFIDENCE LIMITS:	Not Calculable
NO-OBSERVED-EFFECT-CONCENTRATION:	1.9 mg a.i./L
LOWEST-OBSERVED-EFFECT CONCENTRATION:	3.0 mg a.i./L

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INTRODUCTION

This study was conducted by Wildlife International Ltd. for 3M Corporation at the Wildlife International Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the test was conducted from May 24 to May 28, 1999. Raw data generated by Wildlife International Ltd. and a copy of the final report are filed under Project Number 454A-106 in archives located on the Wildlife International Ltd. site.

OBJECTIVE

The objective of this study was to evaluate the acute toxicity of PFOS (Perfluorooctane Sulfonic Acid Potassium Salt) on shell deposition of the eastern oyster, *Crassostrea virginica*, during a 96-hour exposure period under static test conditions.

EXPERIMENTAL DESIGN

Eastern oysters were exposed to a geometric series of five test concentrations and a negative (dilution water) control. One replicate test chamber was maintained in each treatment and control group, with 20 oysters in each test chamber. Nominal test concentrations were selected in consultation with the Sponsor, and were based upon the results of an exploratory range finding toxicity test. Nominal test concentrations selected were 1.2, 2.0, 3.3, 5.5 and 9.1 mg active ingredient (a.i.)/L.. Mean measured test concentrations were determined from samples of test water collected from each treatment and the control group at the beginning of the test, at approximately 48 hours, and at test termination.

The oysters were indiscriminately assigned to exposure chambers at test initiation. Algal cells (*Thalassiosira pseudonana*, *Skeletonema* sp., *Chaetoceros* sp., and *Isochrysis* sp.) were provided to supplement naturally occurring algae and to maximize oyster growth rates during the test.

Measurements of shell deposition for each oyster were made at 96 hours and were used to estimate the EC50 value, the no-observed-effect-concentration (NOEC) and the lowest- observed-effect-concentration (LOEC). The EC50 is the concentration of test substance in water that is estimated to inhibit shell deposition by 50%, relative to the control.

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MATERIALS AND METHODS

The study was conducted based on the procedures outlined in the protocol, "PFOS: A 96-Hour Shell Deposition Test with the Eastern Oyster (*Crassostrea virginica*)". The protocol was based on procedures outlined in U.S. Environmental Protection Agency Series 850 – Ecological Effects Test Guidelines, OPPTS Number 850.1025 (1); *Standard Evaluation Procedure, Acute Toxicity Test for Estuarine and Marine Organisms (Mollusc 96-Hour Flow-Through Shell Deposition Study)* (2) and *ASTM Standard E729-88a Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians* (3).

Test Substance

The test substance was received from 3M Corporation on October 29, 1998 and was assigned Wildlife International Ltd. identification number 4675. The test substance was described as a white powder. It was identified as FC-95 from lot number 217 (T-6295). Information provided by the Sponsor indicated a purity of 98.9%, and an expiration date of 2008. The test substance was reanalyzed by the Sponsor and the Certificate of Analysis dated March 9, 2000 indicated a purity of 90.49%. The test substance was stored at ambient room temperature.

Preparation of Test Concentrations

Nominal test concentrations were 1.2, 2.0, 3.3, 5.5 and 9.1 mg a.i./L, based on a test substance purity of 90.49%. All materials which came into contact with the test substance during preparation of test concentrations were constructed of plastic or stainless steel. A primary stock solution was prepared in dilution water at a concentration of 9.1 mg a.i./L. The primary stock solution was mixed with an electric paddle mixer for approximately 24 hours to aid in the solubilization of the test substance. After mixing, the primary stock solution appeared clear and colorless with some white particulate material suspended throughout the solution. The primary stock was proportionally diluted with dilution water to prepare the four additional test concentrations. All test solutions appeared clear and colorless.

Test Organism

The eastern oyster, *Crassostrea virginica*, was selected as the test species for this study. The Eastern Oyster is representative of an important group of aquatic organisms and was selected for use in the test based upon past history of use and ease of handling in the laboratory. Eastern oysters used in the test were obtained

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from P. Cummins Oyster Company, Baltimore, Maryland. The oysters were held in water from the same source as used during the test. The oysters were supplied unfiltered natural seawater during holding and were held for 12 days prior to test initiation. To supplement the diet of the oysters and enhance their condition and growth, the oysters were provided with an algal suspension of *Thalassiosira* sp., *Skeletonema* sp., *Chaetoceros* sp., and *Isochrysis* sp. continuously during holding and testing.

During the 12-day holding period preceding the test, the oysters showed no signs of disease or stress. During the holding period, water temperatures ranged from 21.8 to 22.9°C. The pH of the water ranged from 7.9 to 8.1, salinity remained at 20 ‰ (parts per thousand) and dissolved oxygen ranged from 7.0 to 7.6 mg/L. Instrumentation used for water measurements is described in the *Environmental Conditions* section of this report.

Prior to test initiation, recently deposited shell at the rounded (ventral) end was removed using a small electric grinder. Care was taken to remove the shell rim uniformly to produce a smooth, rounded, blunt profile. The lengths of 20 impartially-selected oysters were determined by measuring the longest distance from the umbo to the edge of the shell. Measurement was made to the nearest 0.05 mm to confirm that the oysters fell within the 25 to 50 mm size criterion. The average length of the oysters was 33.8 mm with a range of 27.8 to 41.5 mm. At test initiation, the oysters were collected from the holding tank and indiscriminately transferred to the test chambers.

Dilution Water

The water used for holding and testing was natural seawater collected at Indian River Inlet, Delaware, and diluted to a salinity of approximately 20‰ with well water. The dilution water was stored in a 19,000-L tank where it was aerated by recirculation. Salinity and pH measurements taken during the four-week period immediately preceding the test are presented in Appendix I. The results of analyses performed to measure the concentrations of selected contaminants in saltwater used by Wildlife International Ltd. are presented in Appendix II.

Test Apparatus

Test chambers were 52-L polyethylene aquaria containing approximately 40 L of test solution. Each test chamber was continuously stirred to circulate the supplemental algae using an electric paddle mixer. In addition, each test chamber was gently aerated. The depth of water in a representative test chamber was approximately 21 cm. Test chambers were impartially positioned in an environmental chamber set to maintain a temperature of 22±1°C. The test chambers were labeled with the project number and test concentration.

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Environmental Conditions

Lighting used to illuminate the holding tanks and test chambers during holding and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (Colortone® 50). A photoperiod of 16 hours of light and 8 hours of darkness was controlled with an automatic timer. A 30-minute transition period of low light intensity was provided when lights went on and off to avoid sudden changes in lighting. Light intensity at test initiation was approximately 498 lux at the surface of the water. Light intensity was measured using a SPER Scientific Ltd. light meter.

Temperature was measured in each test chamber at the beginning and end of the test using a liquid-in-glass thermometer. Temperature also was measured continuously in the negative control test chamber using a Fulscope ER/C Recorder. The target test temperature during the study was $22 \pm 1^\circ\text{C}$. Dissolved oxygen was measured in each test chamber daily. Measurements of pH were made in each test chamber at test initiation, after approximately 48 hours and at the end of the test. Dilution water salinity was measured at test initiation and test termination.

Measurements of pH were made using a Fisher Accumet Model 915 pH meter, and dissolved oxygen was measured using a Yellow Springs Instrument Model 51B dissolved oxygen meter. Salinity was measured using a Bio-Marine, Inc. Aquafauna refractometer.

Observations

Oysters were observed daily during the test for mortality and clinical signs of toxicity. At the end of the test, the longest finger of new shell growth was measured to the nearest 0.05 mm.

Statistical Analyses

Shell growth inhibition was calculated for each treatment group as the percent reduction in shell growth relative to mean shell growth in the negative control. The following formula was used:

$$\% \text{ Inhibition} = \frac{\text{Mean Shell Growth}_{\text{control}} - \text{Mean Shell Growth}_{\text{treatment}}}{\text{Mean Shell Growth}_{\text{control}}}$$

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The EC50 value was estimated by visual inspection of the shell growth inhibition data. The shell growth data was evaluated for normality and homogeneity of variances using the Chi-Square test and Bartlett's test, respectively. Dunnett's test was then used to identify treatment groups which had a statistically significant (≤ 0.05) reduction in shell growth as compared to the control (4).

Analytical Chemistry

Water samples were collected at mid-depth from each treatment and the control group at the beginning of the test, at approximately 48 hours and at test termination to measure concentrations of the test substance. The samples were collected in plastic (Nalgene®) bottles and analyzed immediately without storage. After review of the analytical results, the samples were reanalyzed in duplicate. Analytical procedures used in the analysis of the samples are provided in Appendix III.

RESULTS AND DISCUSSION

Measurement of Test Concentrations

Results of analyses to measure concentrations of PFOS in water samples collected during the test are presented in Table 1 and in the analytical chemistry report (Appendix III). Nominal concentrations selected for use in this study were 1.2, 2.0, 3.3, 5.5 and 9.1 mg a.i./L. Samples collected at test initiation had measured values that ranged from 28 to 46% of nominal values. After 24 hours of mixing, the primary stock solution was measured to be 32 to 38% of nominal and was probably at the limit of solubility in unfiltered saltwater. The recoveries in the other test concentrations were similar because they were proportional dilutions of the primary stock. Measured values for samples taken at 48 hours ranged from 15 to 41% of nominal. Measured values for samples taken at 96 hours ranged from <LOQ to 52% of nominal. When measured concentrations of the samples analyzed at test initiation, approximately 48 hours and at test termination were averaged, the mean measured concentrations for this study were 0.36, 0.40, 1.3, 1.9 and 3.0 mg a.i./L. Mean measured concentrations were used in the estimation of the EC50 value.

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH are presented in Table 2. Temperatures were within the $22 \pm 1^\circ\text{C}$ range established for the test. Dissolved oxygen concentrations remained ≥ 6.1 mg/L (79 percent of saturation) throughout the test and pH ranged from 7.5 to 8.1. Dilution water salinity measured at test initiation and test termination was 20 and 21‰, respectively.

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Oysters in the negative control and all PFOS treatment groups appeared normal and healthy throughout the test period. After 96 hours of exposure, oyster shell growth in the negative control was 2.67 mm (Table 3). Mean shell growth in the 0.36, 0.40, 1.3, 1.9 and 3.0 mg a.i./L treatment groups was 2.50, 2.40, 2.51, 2.13 and 1.91 mm, respectively. When compared to the negative control group, shell growth inhibition ranged from 6.4% in the 0.36 mg a.i./L treatment group to 28% in the 3.0 mg a.i./L treatment group. Dunnett's test showed that oyster shell growth was significantly reduced in the 3.0 mg a.i./L treatment group in comparison to the negative control. Individual measurements of oyster shell growth are presented in Table 4.

CONCLUSIONS

The 96-hour EC50 value for eastern oysters (*Crassostrea virginica*) exposed to PFOS was >3.0 mg a.i./L, the highest concentration tested and the practical limit of solubility in unfiltered saltwater. Based on a statistically significant reduction in shell deposition in the 3.0 mg a.i./L treatment group, the LOEC was 3.0 mg a.i./L and the NOEC was 1.9 mg a.i./L.

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REFERENCES

- 1 **U.S. Environmental Protection Agency.** 1996. Series 850-Ecological Effects Test Guidelines (draft), OPPTS Number 850.1025: *Oyster Acute Toxicity Test (Shell Deposition)*.
- 2 **U.S. Environmental Protection Agency.** 1985. *Standard Evaluation Procedure, Acute Toxicity Test for Estuarine and Marine Organisms (Mollusc 96-Hour Flow-Through Shell Deposition Study)*. EPA 540/9-85-011. Washington, D.C.
- 3 **ASTM Standard E729-88a.** 1994. *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians*. American Society for Testing and Materials.
- 4 **West, Inc. and D. D. Gulley.** TOXSTAT Version 3.5. Copyright 1996. Western EcoSystems Technology, Inc., Cheyenne, Wyoming.

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Table 1

Summary of Analytical Chemistry Data

Sponsor:	3M Corporation			
Test Substance:	PFOS			
Test Organism:	Eastern Oyster, <i>Crassostrea virginica</i>			
Dilution Water:	Unfiltered Saltwater			
Nominal Test Concentration (mg a.i./L)	Sampling Time (Hours)	Measured Concentration (mg a.i./L)	Mean Measured Concentration (mg a.i./L)	Percent Of Nominal
Negative Control	0	<LOQ ¹	<LOQ	--
	0	<LOQ		
	48	<LOQ		
	48	<LOQ		
	96	<LOQ		
	96	<LOQ		
1.2	0	0.331	0.36 ²	30
	0	0.353		
	48	0.341		
	48	0.429		
	96	<LOQ		
	96	<LOQ		
2.0	0	0.622	0.40	20
	0	0.633		
	48	0.299		
	48	0.313		
	96	0.249		
	96	0.257		
3.3	0	1.36	1.3	39
	0	1.15		
	48	0.924		
	48	0.878		
	96	1.58		
	96	1.72		
5.5	0	2.42	1.9	35
	0	2.53		
	48	2.02		
	48	2.24		
	96	1.45		
	96	0.970		
9.1	0	3.44	3.0	33
	0	3.01		
	48	3.74		
	48	3.57		
	96	1.99		
	96	2.19		

¹The limit of quantitation (LOQ) was 0.115 mg a.i./L.²Mean measured concentration does not include values <LOQ.

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Table 2

Temperature, Dissolved Oxygen and pH of Water in the Test Chambers

Sponsor:	3M Corporation										
Test Substance:	PFOS										
Test Organism:	Eastern Oyster, <i>Crassostrea virginica</i>										
Dilution Water:	Unfiltered Saltwater										
Mean Measured	0 Hour			24 Hours		48 Hours		72 Hours		96 Hours	
Test Concentration	Temp ¹	DO ²		DO	DO		DO		Temp	DO	
(mg a.i./L.)	(°C)	(mg/L)	pH	(mg/L)	(mg/L)	pH	(mg/L)		(°C)	(mg/L)	pH
Negative Control	22.3	7.6	8.1	6.8	6.9	7.7	6.6		22.2	6.8	7.6
0.36	22.3	7.6	8.1	6.6	6.7	7.7	6.5		22.1	6.7	7.6
0.40	22.4	7.6	8.1	6.4	6.6	7.7	6.3		22.1	6.7	7.6
1.3	22.6	7.6	8.1	6.4	6.6	7.7	6.3		22.1	6.6	7.6
1.9	22.6	7.6	8.1	6.4	6.7	7.7	6.3		22.0	6.3	7.5
3.0	22.7	7.7	8.1	6.4	6.5	7.7	6.1		21.8	6.4	7.6

¹ Temperature measured continuously during the test ranged from approximately 22.0 to 22.5°C.² A dissolved oxygen concentration of 4.7 mg/L represents 60% saturation.

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Table 3

Shell Deposition and Shell Growth Inhibition at Test Termination

Sponsor: 3M Corporation		
Test Substance: PFOS		
Test Organism: Eastern Oyster, <i>Crassostrea virginica</i>		
Dilution Water: Unfiltered Saltwater		
Mean Measured Concentration (mg a.i./L)	Shell Deposition (mm) Mean \pm SD ¹	Shell Growth Inhibition (%)
Negative Control	2.67 \pm 0.824	--
0.36	2.50 \pm 0.933	6.4
0.40	2.40 \pm 0.820	10
1.3	2.51 \pm 0.919	6.0
1.9	2.13 \pm 0.804	20
3.0	1.91 ² \pm 0.591	28
The 96-hour EC50 value was >3.0 mg a.i./L.		
¹ Mean and standard deviation for 20 oysters.		
² Indicates a significant difference from the negative control using Dunnett's test ($p \leq 0.05$).		

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Table 4

Individual Oyster Shell Measurements

Sponsor: 3M Corporation						
Test Substance: PFOS						
Test Organism: Eastern Oyster, <i>Crassostrea virginica</i>						
Dilution Water: Unfiltered Saltwater						
Mean Measured Concentrations (mg a.i./L)						
	Negative Control	0.36	0.40	1.3	1.9	3.0
1	4.00	2.05	3.20	1.50	2.20	1.65
2	3.25	1.80	2.25	2.80	1.45	0.80
3	3.00	1.90	3.10	2.65	3.60	2.20
4	2.55	3.65	0.90	2.25	1.80	1.75
5	2.50	2.55	2.35	2.40	2.25	3.05
6	2.75	1.90	2.10	2.70	2.30	1.60
7	2.70	2.40	2.25	2.90	3.10	0.90
8	2.70	4.00	2.80	4.45	0.65	2.40
9	1.85	1.45	1.75	1.80	0.80	2.25
10	2.20	2.55	2.45	3.05	2.40	1.80
11	4.40	3.55	2.85	1.60	2.70	2.70
12	2.05	1.80	3.00	0.85	1.65	1.10
13	1.55	3.35	4.45	4.10	1.80	2.35
14	3.05	2.30	3.05	2.70	2.20	1.70
15	2.45	3.70	1.95	2.45	1.70	2.15
16	4.20	3.75	1.20	3.00	3.15	2.60
17	1.45	2.60	1.70	1.85	0.90	1.60
18	1.75	0.90	2.05	2.95	2.20	1.45
19	2.85	2.85	1.65	0.95	2.90	2.00
20	2.20	0.95	2.90	3.30	2.85	2.20
Mean	2.67	2.50	2.40	2.51	2.13	1.91
SD	0.824	0.933	0.820	0.919	0.804	0.591
Range	1.45 - 4.40	0.90 - 4.00	0.90 - 4.55	0.85 - 4.45	0.65 - 3.60	0.80 - 3.05

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APPENDIX I

Salinity and pH of Unfiltered Saltwater Measured
During the 4-Week Period Immediately Preceding the Test

Sponsor:	3M Corporation	
Test Substance:	PFOS	
Test Organism:	Eastern Oyster, <i>Crassostrea virginica</i>	
Dilution Water:	Unfiltered Saltwater	

	Mean	Range
Salinity (‰)	21 (N = 4)	20 – 21
pH	8.1 (N = 4)	8.0 – 8.2

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APPENDIX II
Analyses of Pesticides, Organics, Metals and Other Inorganics
in Wildlife International Ltd. Saltwater¹

ANALYSIS	MEASURED CONCENTRATION	
Miscellaneous Measurements		
Total Dissolved Solids	23,500	mg/L
Ammonia Nitrogen	<	0.050 mg/L
Total Organic Carbon ²	<	1.0 mg/L
Total Cyanide	<	10.0 µg/L
Organochlorines and PCBs		
Aldrin	<	0.005 µg/L
Alpha BHC	<	0.005 µg/L
Beta BHC	<	0.005 µg/L
Delta BHC	<	0.005 µg/L
Gamma BHC (Lindane)	<	0.006 µg/L
Chlordane	<	0.025 µg/L
DDD, pp'	<	0.006 µg/L
DDE, pp'	<	0.005 µg/L
DDT, pp'	<	0.008 µg/L
Dieldrin	<	0.005 µg/L
Endosulfan, A	<	0.005 µg/L
Endosulfan, B	<	0.005 µg/L
Endosulfan Sulfate	<	0.018 µg/L
Endrin	<	0.010 µg/L
Endrin Aldehyde	<	0.005 µg/L
Heptachlor	<	0.005 µg/L
Methoxychlor	<	0.007 µg/L
Heptachlor Epoxide	<	0.005 µg/L
Toxaphene	<	0.500 µg/L
PCB-1016	<	0.260 µg/L
PCB-1221	<	0.260 µg/L
PCB-1232	<	0.260 µg/L
PCB-1242	<	0.720 µg/L
PCB-1248	<	0.720 µg/L
PCB-1254	<	0.720 µg/L
PCB-1260	<	0.720 µg/L
Metals and Other Inorganics		
Aluminum ³	<	100 µg/L
Arsenic ³	<	25.0 µg/L
Beryllium ³	<	0.50 µg/L
Cadmium ³	<	1.0 µg/L
Calcium ³	<	235 mg/L
Chromium ³	<	2.0 µg/L
Cobalt ³	<	1.0 µg/L
Copper ³	<	20.0 µg/L
Iron ³	<	100 µg/L
Lead ³	<	10.0 µg/L
Magnesium ³	<	760 mg/L
Manganese ³	<	4.0 µg/L
Mercury	<	0.20 µg/L
Molybdenum ³	<	2.0 µg/L
Nickel ³	<	20.0 µg/L
Potassium ³	<	277 mg/L
Selenium ³	<	25.0 µg/L
Silver ³	<	1.0 µg/L
Sodium ³	<	6,010 mg/L
Zinc ³	<	20.0 µg/L

¹Analyses performed by QST Environmental, Gainesville, Florida for samples collected on November 3 through November 7, 1997.²Analyses performed by Wildlife International Ltd. for the sample collected on November 5, 1997.³Analyses performed by Wildlife International Ltd. for samples collected on November 5 through 7, 1997.

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APPENDIX III

THE ANALYSIS OF PFOS IN UNFILTERED SALTWATER
IN SUPPORT OF
WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 454A-106

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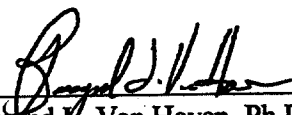
REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFOS: A 96-Hour Shell Deposition Test with the Eastern Oyster (*Crassostrea virginica*)

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 454A-106

PRINCIPAL INVESTIGATOR:




Raymond E. Van Hoven, Ph.D.
Scientist

1-25-00

DATE

MANAGEMENT:



Willard B. Nixon, Ph.D.
Manager, Analytical Chemistry

4/26/00

DATE

000766

AMENDED

Introduction

Unfiltered saltwater samples were collected from an acute toxicity study designed to determine the effects of PFOS (Perfluorooctane Sulfonic Acid Potassium Salt) on the shell deposition of the eastern oyster (*Crassostrea virginica*). This study was conducted by Wildlife International Ltd. and identified as Project No.: 454A-106. The analyses of these water samples were performed at Wildlife International Ltd. using high performance liquid chromatography with mass spectrometric detection (HPLC/MS). Samples were received for analysis on May 24, 26 and 28, 1999. Samples were analyzed on each sample receipt day. All samples were reanalyzed on June 6, 1999 to confirm original analyses. The second analysis was reported.

Test Substance and Internal Standard

The test substance used for the analytical portion of this study was Wildlife International Ltd. identification number 4675. The test substance was used to prepare calibration standards and matrix fortification samples.

The internal standard was received from 3M Corporation on July 2, 1998 and was assigned Wildlife International Ltd. identification number 4526 upon receipt. The internal standard, a granular material, was identified as: 1H, 1H, 2H, 2H Perfluorooctane Sulfonic Acid, Chemical Abstract Number: 27619-97-2. The standard was stored under ambient conditions.

Analytical Method

The method used for the analysis of the unfiltered saltwater samples was developed at Wildlife International Ltd. and entitled "Analytical Method Validation for the Determination of PFOS in Freshwater, Saltwater, and Algal Media". This methodology was included as Appendix II of Wildlife International Ltd. protocol number 454/011299/MVAL/SUB454. It was based upon methodology provided by 3M Corporation.

Samples were diluted in a 50% methanol : 50% NANOpure® water solution containing 0.100 mg 4H PFOS (internal standard)/L and 0.05% formic acid (v/v) so that they fell within the calibration range of the PFOS methodology.

Concentrations of PFOS in the standards and samples were determined by reversed-phase high performance liquid chromatography using a Hewlett-Packard Model 1100 High Performance Liquid Chromatograph (HPLC) with a Perkin-Elmer API 100LC Mass Spectrometer equipped with a Perkin-Elmer TurboIonSpray ion source. HPLC separations were achieved using a Keystone Betasil C₁₈ analytical column (100 mm x 2 mm I.D., 3 µm particle size). The instrument parameters are summarized in Table 1. A method flowchart is provided in Figure 1.

Calibration Curve and Limit of Quantitation

Calibration standards of PFOS prepared in a 50% methanol : 50% NANOpure® water solution containing 0.100 mg 4H PFOS (internal standard)/L and 0.05% formic acid (v/v), ranging in concentration from 0.00229 to 0.0274 mg a.i./L were analyzed with the samples. Linear regression equations were generated using peak area response ratios (PFOS : internal standard) versus the respective concentration ratios (PFOS : internal standard) of the calibration standards. A typical calibration curve is presented in Figure 2. The concentration of PFOS in the samples was determined by substituting the peak area response ratios into the applicable linear regression equation. Representative ion chromatograms of low and high calibration standards are presented in Figures 3 and 4, respectively.

The method limit of quantitation (LOQ) for these analyses was set at 0.115 mg a.i./L calculated as the product of the lowest calibration standard analyzed (0.00229 mg a.i./L) and the dilution factor of the matrix blank samples (50.0).

Matrix Blank and Fortification Samples

One matrix blank sample was analyzed to determine possible interference. No interferences were observed at or above the LOQ during samples analyses (Table 2). The matrix blank chromatogram is presented in Figure 5.

Unfiltered saltwater was fortified at 0.457, 4.57 and 11.0 mg a.i./L and analyzed concurrently with the samples (Table 2). Sample concentrations were not corrected for mean procedural recovery. High recoveries were obtained for the low-level (0.457 mg a.i./L) matrix fortification sample. Previous analyses in saltwater verified that the method is capable of producing quantitative recoveries at this concentration level. A representative chromatogram of a matrix fortification is presented in Figure 6.

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Example Calculations

Sample number 454A-106-5B, nominal concentration of 5.5 mg a.i./L in unfiltered saltwater.

Initial Volume: 0.100 mL

Final Volume: 25.0 mL

Dilution Factor: 250

PFOS Peak Area: 71220

Internal Standard Peak Area: 74271

Peak Area Ratio: 0.95892

Calibration curve equation.

Slope: 7.78372

Intercept: 0.17175

Curve is weighted (1/x).

$$\begin{aligned}\text{PFOS (mg a.i./L) at instrument} &= \frac{(\text{Peak area ratio} - (\text{Y-intercept})) \times \text{I.S. Concentration}}{\text{Slope}} \\ &= \frac{(0.95892 - 0.17175) \times 0.100 \text{ mg a.i./L}}{7.78372} \\ &= 0.0101 \text{ mg a.i./L}\end{aligned}$$

Note: I.S. = internal standard.

$$\begin{aligned}\text{PFOS (mg a.i./L) in sample} &= \frac{\text{PFOS (mg a.i./L) at instrument} \times \text{Final Volume (mL)}}{\text{Initial Volume (mL)}} \\ &= \frac{0.0101 \times 25.0}{0.100} \\ &= 2.53 \text{ mg a.i./L}\end{aligned}$$

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$$\text{Percent of Nominal Concentration} = \frac{\text{PFOS (mg a.i./L) in sample}}{\text{PFOS (mg a.i./L) nominal}} \times 100$$

Calculated recovery: 46.1%

Note: manual calculation may differ.

RESULTS

Sample Analysis

Unfiltered saltwater samples were collected from the shell deposition test with the eastern oyster (*Crassostrea virginica*) at test initiation, May 24, 1999 (Hour 0), on May 26, 1999 (Hour 48), and at test termination, May 28, 1999 (Hour 96). The measured concentrations of PFOS in the samples collected at initiation of exposure of the test organisms (Hour 0) ranged from 27.9 to 46.1% of the nominal concentrations. Samples collected at Hour 48 had a measured concentration range of 14.9 to 40.9% of nominal values. Samples collected at test termination (Hour 96) had a measured concentration range of < LOQ to 52.2% of nominal values (Table 3). A representative chromatogram of a test sample is shown in Figure 7.

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Table 1

Typical LC/MS Operational Parameters

INSTRUMENT:	Hewlett-Packard Model 1100 High Performance Liquid Chromatograph with a Perkin-Elmer API 100LC Mass Spectrometer equipped with a Perkin-Elmer TurboIonSpray ion source. Operated in selective ion monitoring mode (SIM).
ANALYTICAL COLUMN:	Keystone Betasil C ₁₈ column (50 mm x 2 mm I.D., 3 µm particle size)
OVEN TEMPERATURE:	30°C
STOP TIME:	5.00 minutes
FLOW RATE:	0.220 mL/minute
MOBILE PHASE:	72.0% Methanol : 28.0% NANOpure® Water containing 0.1% Formic Acid
INJECTION VOLUME:	25.0 µL
PFOS RETENTION TIME:	Approximately 3.6 minutes
INTERNAL STANDARD RETENTION TIME:	Approximately 2.6 minutes
PFOS MONITORED MASS:	498.6 amu
INTERNAL STANDARD MONITORED MASS:	426.7 amu

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Table 2

Matrix Blank and Fortifications Analyzed Concurrently During Sample Analysis

Sample		Concentrations of PFOS (mg a.i./L)		Percent Recovered
Number (454-106-)	Type	Fortified	Measured ¹	
MAB-4	Matrix Blank	0.0	<LOQ	--
MAS-10	Matrix Fortification	0.457	0.760	166
MAS-10A ²	Matrix Fortification	0.457	0.812	177
MAS-10B ²	Matrix Fortification	0.457	0.769	168
MAS-11	Matrix Fortification	4.57	4.57	99.8
MAS-12	Matrix Fortification	11.0	13.0	119

Note: Results and corrections for new test substance purity were generated using MacQuan version 1.5 software and manual calculations. Values have been rounded for reporting purposes.

¹ The limit of quantitation (LOQ) was 0.115 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.00229 mg a.i./L) and the dilution factor of the matrix blank samples (50.0).

² Sample 454-106-MAS-10 was rediluted in duplicate and analysis confirmed original result; suspect fortification error.

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Table 3

Measured Concentrations of PFOS in Unfiltered Saltwater Samples from an Oyster Shell Deposition Test

Nominal Test Concentration (mg a.i./L)	Sample Number (454A-106-)	Sampling Time (Hours)	PFOS Measured Concentration ¹ (mg a.i./L)	Percent of Nominal
0.0	1A	0	< LOQ	—
	1B	0	< LOQ	—
	7A	48	< LOQ	—
	7B	48	< LOQ	—
	13A	96	< LOQ	—
	13B	96	< LOQ	—
1.2	2A	0	0.331	27.9
	2B	0	0.353	29.7
	8A	48	0.341	28.7
	8B	48	0.429	36.1
	14A	96	< LOQ	—
	14B	96	< LOQ	—
2.0	3A	0	0.622	30.9
	3B	0	0.633	31.5
	9A	48	0.299	14.9
	9B	48	0.313	15.6
	15A	96	0.249	12.4
	15B	96	0.257	12.8
3.3	4A	0	1.36	41.4
	4B	0	1.15	34.9
	10A	48	0.924	28.0
	10B	48	0.878	26.7
	16A	96	1.58	48.0
	16B	96	1.72	52.2
5.5	5A	0	2.42	44.1
	5B	0	2.53	46.1
	11A	48	2.02	36.9
	11B	48	2.24	40.9
	17A	96	1.45	26.4
	17B	96	0.970	17.6
9.1	6A	0	— ²	—
	6B	0	3.39	37.1
	6C	0	3.44	37.6
	6D	0	3.01	32.9
	12A	48	3.74	40.9
	12B	48	3.57	39.0
	18A	96	1.99	21.7
	18B	96	2.19	23.9

Note: Results and corrections for new test substance purity were generated using MacQuan version 1.5 software and manual calculations. Values have been rounded for reporting purposes.

¹ The limit of quantitation (LOQ) was 0.115 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.00229 mg a.i./L) and the dilution factor of the matrix blank samples (50.0).

² Result for sample 6A (6.21 mg a.i./L) was not included in the table. Sample 6 was rediluted in duplicate (6C and 6D) and results of these samples confirmed result for sample 6B.

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**METHOD OUTLINE FOR THE ANALYSIS OF PFOS
IN UNFILTERED SALTWATER**

Prepare matrix fortification samples by spiking the requisite volume of PFOS stock solutions directly into unfiltered saltwater using gas-tight syringes and Class A volumetric flasks.



Dilute matrix fortification and test samples into the range of the calibration standards by partially filling Class A volumetric flasks with 50% methanol : 50% NANOpure® water solution containing 0.100 mg 4H PFOS (internal standard)/L and 0.05% formic acid (v/v). Add the appropriate volume of sample and bring the flask to volume with the dilution solvent. Process the matrix blank sample using the same dilution and aliquot volume as for the lowest fortification level. Mix well by several repeat inversions.



Ampulate samples and submit for LC/MS analysis.

Figure 1. Analytical method flowchart for the analysis of PFOS in unfiltered saltwater.

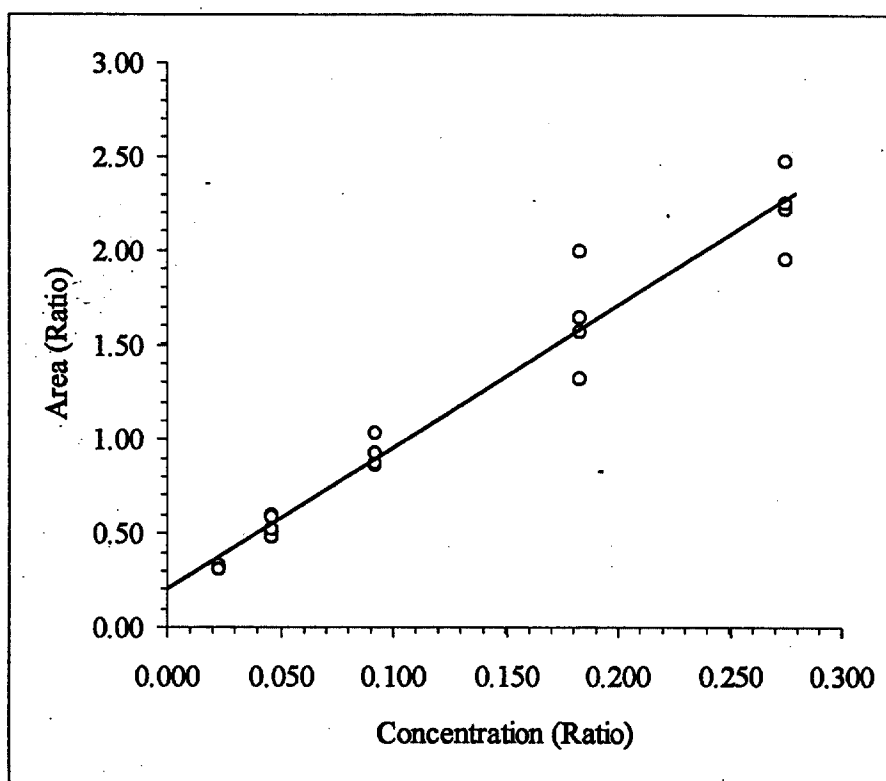


Figure 2. A typical calibration curve for PFOS. Slope = 7.78372; Intercept = 0.17175; $r = 0.9825$. Curve is weighted ($1/x$).

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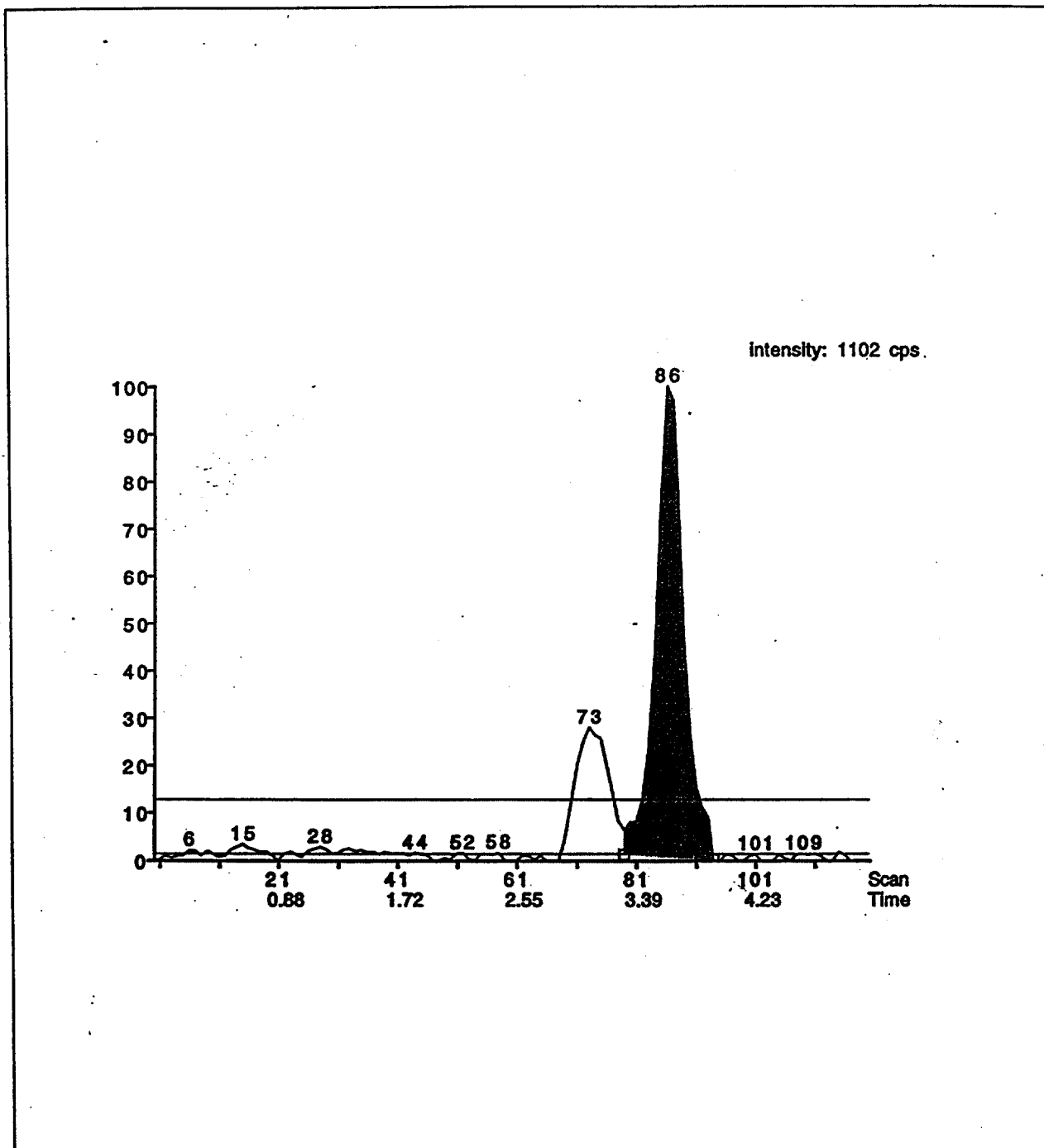


Figure 3. A representative ion chromatogram of a low-level (0.00229 mg a.i./L) PFOS standard.

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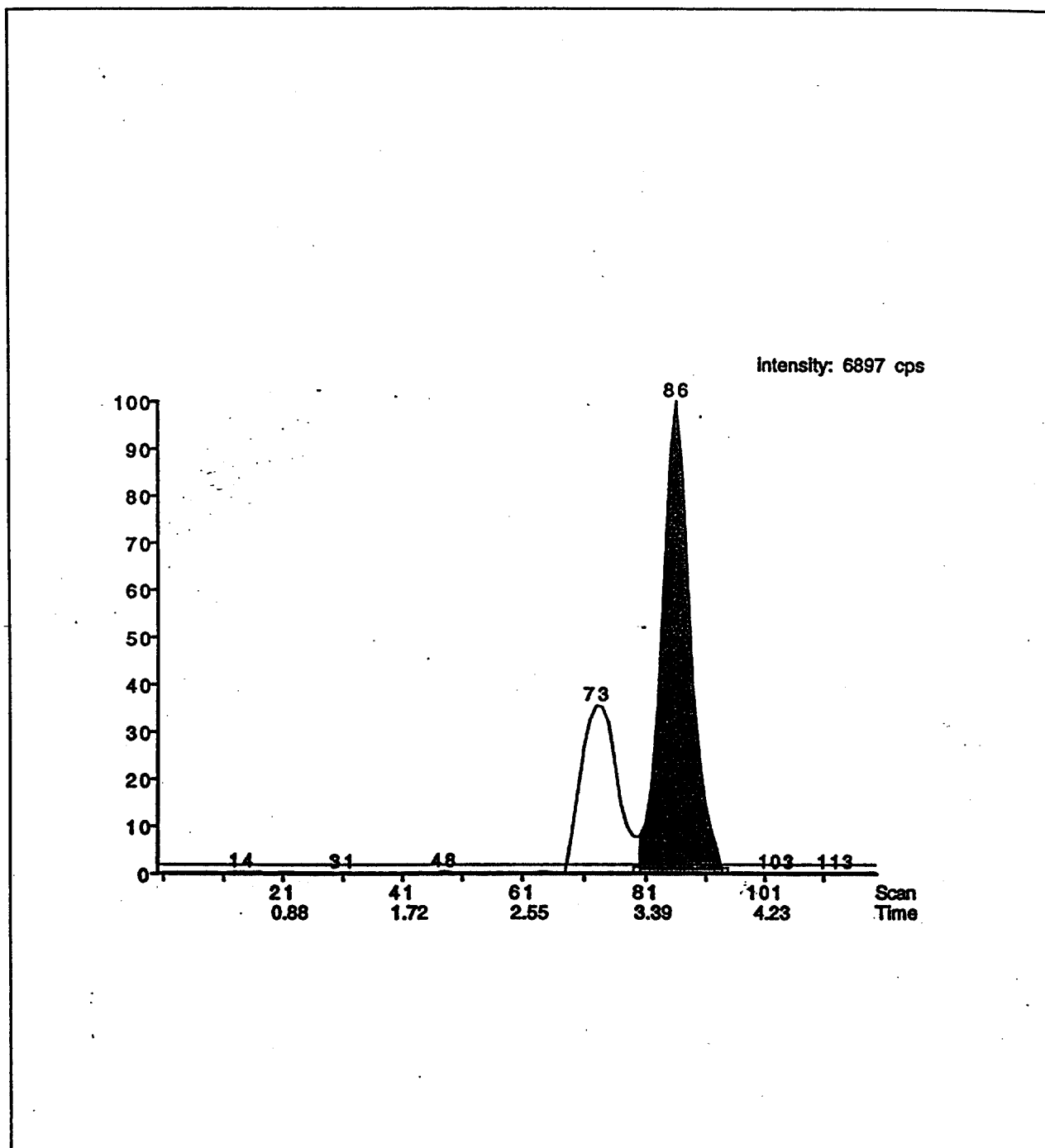


Figure 4. A representative ion chromatogram of a high-level (0.0274 mg a.i./L) PFOS standard.

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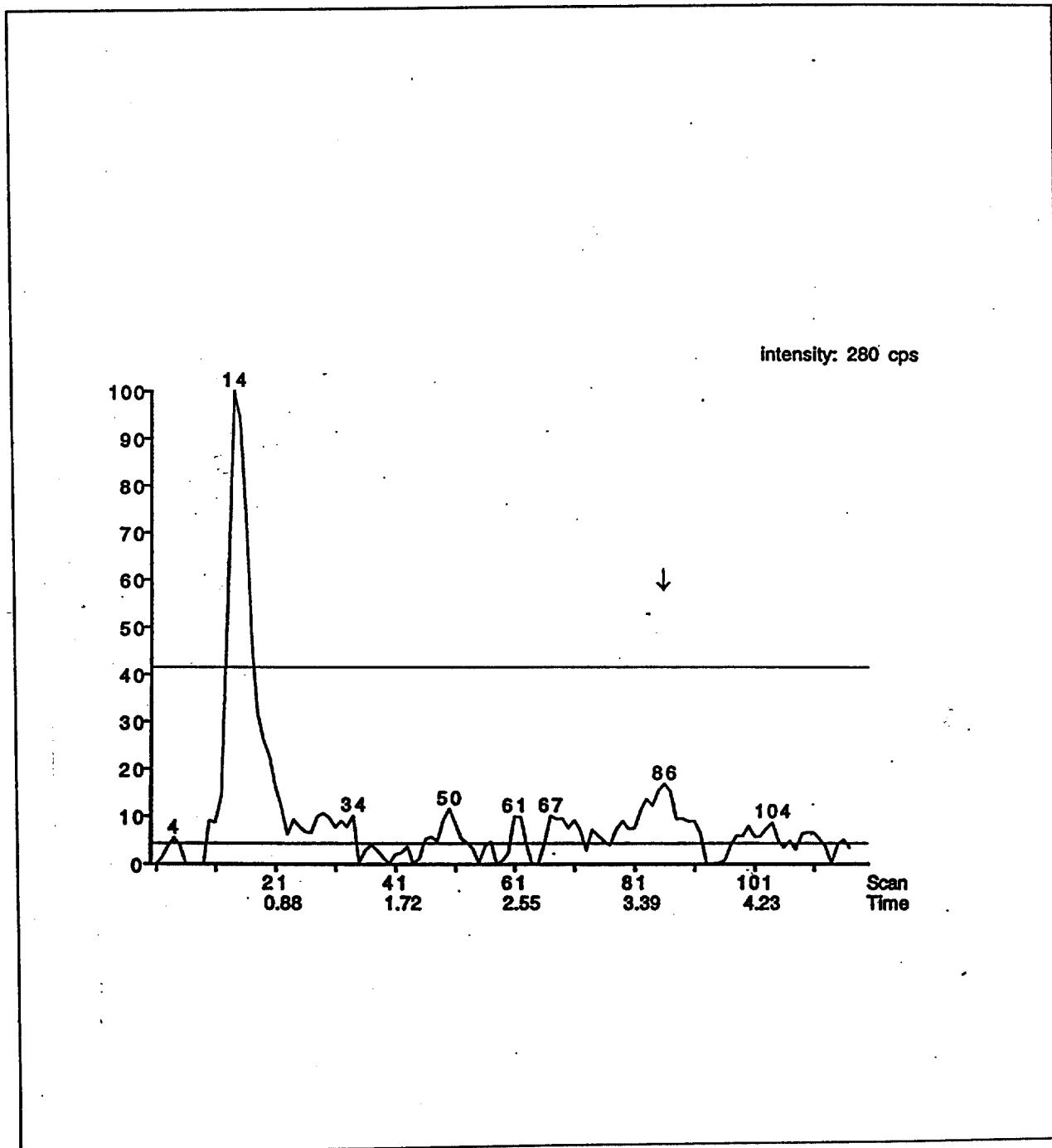


Figure 5. The matrix blank ion chromatogram (454A-106-MAB-4). The arrow indicates the retention time of PFOS.

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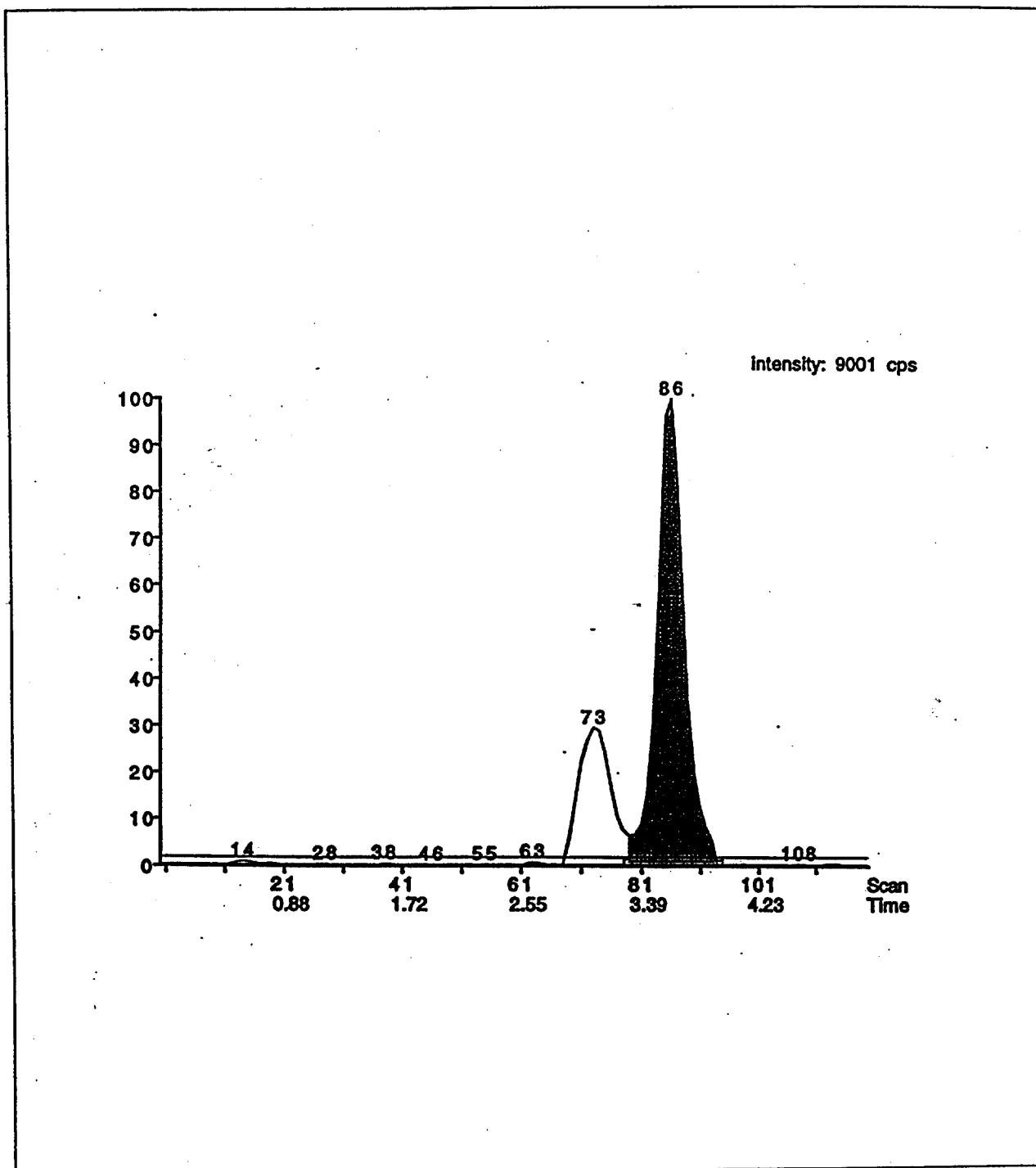


Figure 6. A representative ion chromatogram of a 4.57 mg a.i./L matrix fortification sample (454A-106-MAS-11).

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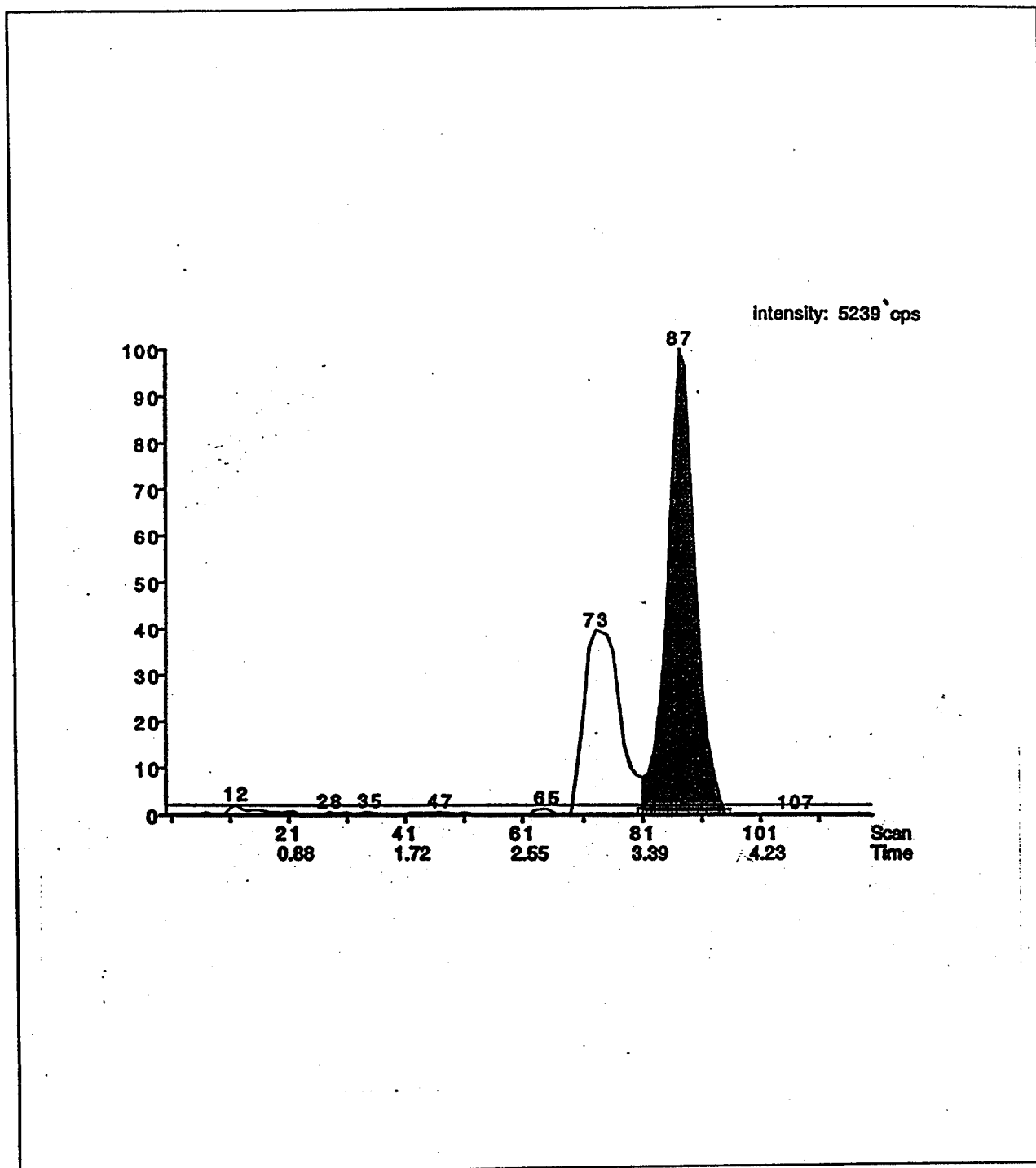


Figure 7. A representative ion chromatogram of a 5.5 mg a.i./L test sample (454A-106-5B).

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AMENDED

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APPENDIX IV

Changes to Protocol

This study was conducted in accordance with the approved Protocol with the following changes:

1. The protocol was amended to add the proposed experimental start and termination dates, test concentrations and test substance number.
2. The protocol was amended to specify that GLP analyses of saltwater will be reported.
3. The protocol was amended to change the analytical sampling schedule.
4. Salinity was measured in the negative control at test initiation and termination.

APPENDIX V

Personnel Involved in the Study

The following key Wildlife International Ltd. personnel were involved in the conduct or management of this study:

1. Henry O. Krueger, Ph.D., Director, Aquatic Toxicology and Non-Target Plants
2. Willard B. Nixon, Ph.D., Manager, Analytical Chemistry
3. Mark A. Mank, Laboratory Supervisor
4. Timothy Z. Kendall, Laboratory Supervisor
5. Kurt R. Drott, Senior Biologist
6. Raymond L. VanHoven, Ph.D., Scientist

APPENDIX VI

Report Amendment

1. Original Report: Pages 1-4, 6 and 22
Amendment: The pages were changed to include the amended report date, revised page numbers, and new signatures and dates due to the addition of the report amendment as Appendix VI.
Reason: To reflect the issuing of an amended report.
2. Original Report: Page 2
Amendment: The compliance statement was revised.
Reason: To clarify how the test substance was characterized.
3. Original Report: Page 9
Amendment: Information provided by the Sponsor reflecting the reanalysis of the test substance, including the reanalysis date and the purity, was added to the Test Substance section.
Reason: To reflect the current test substance information provided by the Sponsor.
4. Original Report: Entire report
Amendment: All test substance concentrations were changed to reflect the purity of the test substance as determined by the Sponsor in a reanalysis of the test substance (FC-95, Lot 217). Test concentrations originally were based on the reported purity of 98.9%. The certificate of analysis dated March 9, 2000 indicated a purity of 90.49%. Therefore, all test substance concentrations, including nominal concentrations, measured concentrations, and LC50 values, were recalculated and reported as mg a.i./L based on the 90.49% purity.
Reason: To report the results of the test based on the test substance purity of 90.49% at the request of the Sponsor.
5. Original Report: Page 15
Amendment: Add footnotes 1 and 2 to Table 1, Summary of Analytical Chemistry Data.
Reason: To clarify the measured concentrations reported.

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APPENDIX VI

-Continued-

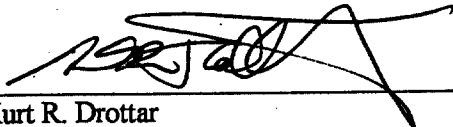
Report Amendment


6. Original Report: Page 17

Amendment: Change the 96-hour EC50 value of >10 mg a.i./L to >3.0 mg a.i./L.

Reason: To correct the reported EC50 value to reflect the mean measured concentration, rather than the nominal concentration.

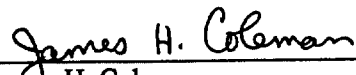
AMENDMENT SIGNATURES:


Kurt R. Drott
Study Director4/26/00

DATE
Henry O. Krueger, Ph.D.
Director, Aquatic Toxicology and Non-Target Plants4/26/00

DATE

REVIEWED BY:


James H. Coleman
Quality Assurance4-26-00

DATE

000784

AMENDED

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: PFOS: A 96-HOUR SHELL DEPOSITION TEST WITH THE EASTERN OYSTER (*Crassostrea virginica*)

PROTOCOL NO.: 454/042199/OYS-DEP/SUB454

AMENDMENT NO.: 1

SPONSOR: 3M Corporation

PROJECT NO.: 454A-106

EFFECTIVE DATE: May 20, 1999

AMENDMENT: Page 2

Add: Experimental Start Date: 5/24/99

Experimental Termination Date: 5/28/99

Test Concentrations: Negative Control, 1.3, 2.2, 3.6, 6.0 and 10 mg a.i./L

Test Substance No.: 4675

REASON: The above information was not known when the protocol was signed by the Study Director.

AMENDMENT: Dilution Water, Page 5

Change: Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents of the seawater and the results of the analyses will be summarized in the final report.

To: Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents of the seawater and the results of the most recent GLP compliant analyses will be summarized in the final report.

REASON: To specify that GLP analyses will be reported.

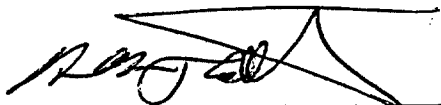
AMENDMENT: Sampling for analytical Measurements, Page 7

Change: Water samples will be collected from each test chamber at test initiation and every 24 hours (± 1 hour) during the test to determine concentrations of the test substance.

To: Water samples will be collected from each test chamber at test initiation, at the midpoint of the test (approximately 48 hours) and at test termination to determine concentrations of the test substance.

REASON: The Sponsor requested a change in the analytical sampling schedule.

000785



STUDY DIRECTOR

5/24/99

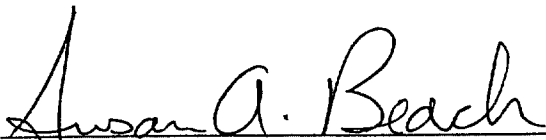
DATE



LABORATORY MANAGEMENT

5/24/99

DATE



SPONSOR'S REPRESENTATIVE

6/7/99

DATE

000786

DEVIATION TO STUDY PROTOCOL

STUDY TITLE: PFOS: A 96-HOUR SHELL DEPOSITION TEST WITH THE EASTERN OYSTER (*Crassostrea virginica*)

PROTOCOL NO.: 454/042199/OYS-DEP/SUB454

DEVIATION NO.: 1

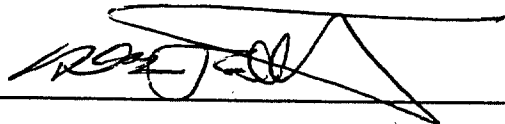
SPONSOR: 3M Corporation

PROJECT NO.: 454A-106

DATE OF DEFACTO DEVIATION: May 24, 1999

DEVIATION: The protocol states that dilution water salinity will be measured in each test chamber at test initiation and at the end of the test. Dilution water salinity was only measured in the negative control at the beginning and end of the test.

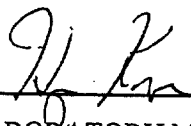
REASON: Biologist oversight. Wildlife International Ltd. stores unfiltered saltwater in a 5,000 gallon tank which is recirculated to maintain constant salinity. Consequently, it is the best judgment of the Study Director that this deviation did not adversely affect the results of the study.



STUDY DIRECTOR

6/17/99

DATE



LABORATORY MANAGEMENT

6/17/99

DATE

PROTOCOL

PFOS: A 96-HOUR SHELL DEPOSITION TEST
WITH THE EASTERN OYSTER (*Crassostrea virginica*)

U.S. Environmental Protection Agency
Series 850 - Ecological Effects Test Guidelines
OPPTS Number 850.1025

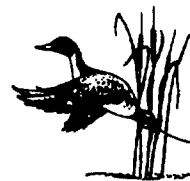
3M Lab Request No. U2723

Submitted to

3M Corporation
Environmental Laboratory
Building 2-3E-09
935 Bush Avenue
St. Paul, Minnesota 55144



WILDLIFE INTERNATIONAL LTD.



8598 Commerce Drive
Easton, Maryland 21601
(410) 822-8600

April 21, 1999

000788

PFOS: A 96-HOUR SHELL DEPOSITION TEST
WITH THE EASTERN OYSTER (*Crassostrea virginica*)

SPONSOR: 3M Corporation
Environmental Laboratory
Building 2-3E-09
935 Bush Avenue
St. Paul, Minnesota 55144

SPONSOR'S REPRESENTATIVE: Ms. Susan A. Beach

TESTING FACILITY: Wildlife International, Ltd.
8598 Commerce Drive
Easton, Maryland 21601

STUDY DIRECTOR: Kurt Drottar
Senior Aquatic Biologist

LABORATORY MANAGEMENT: Henry O. Krueger, Ph.D.
Director of Aquatic Toxicology & Non-Target Plants

FOR LABORATORY USE ONLY

Proposed Dates:

Experimental

Start Date: _____

Experimental

Termination Date: _____

Project No.: 454A-106

Test Concentrations: _____

Test Substance No.: _____

Reference Substance No. (if applicable): _____

PROTOCOL APPROVAL


STUDY DIRECTOR

5/3/99
DATE


LABORATORY MANAGEMENT

5/3/99
DATE


SPONSOR'S REPRESENTATIVE

4/29/99
DATE

000789

INTRODUCTION

Wildlife International, Ltd. will conduct a static acute toxicity test with the Eastern oyster (*Crassostrea virginica*) for the Sponsor at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The study will be performed based on procedures in the U.S. Environmental Protection Agency Series 850 - Ecological Effects Test Guidelines OPPTS Number 850.1025 (1); *Standard Evaluation Procedure, Acute Toxicity Test for Estuarine and Marine Organisms (Mollusc 96-Hour Flow-Through Shell Deposition Study)* (2) and *ASTM Standard E729-88a Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians* (3). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site, or at an alternative location to be specified in the final report.

PURPOSE

The purpose of this study is to determine the effects of a test substance on the shell deposition of the eastern oyster (*Crassostrea virginica*) during a 96-hour exposure under static test conditions.

EXPERIMENTAL DESIGN

Oysters will be exposed to a geometric series of at least five test concentrations and a negative (dilution water) control for 96 hours. One test chamber will be maintained for each treatment and control group with 20 oysters in each chamber. Nominal test concentrations will be selected in consultation with the Sponsor, and will be based upon information such as the results of exploratory range-finding toxicity data, known toxicity data, physical/chemical properties of the test substance or other relevant information. Target concentrations will not exceed 120 mg/L or the solubility limit of the test substance in water (whichever is lower). Generally, each test substance concentration used in the definitive test will be at least 60% of the next higher concentration unless information concerning the concentration-effect curve indicates that a different dilution factor would be more appropriate. Water samples from appropriate test chambers will be collected at specified intervals for analysis of the test substance. Results of analyses will be used to calculate mean measured test concentrations.

Oysters approximately 25 to 50 mm long will be maintained at Wildlife International, Ltd. in unfiltered saltwater for a period of at least 10 days. To control bias, oysters used in the test will be indiscriminately distributed. No other potential sources of bias are expected to affect the results of the study.

Just prior to the initiation of the test, recently deposited shell will be removed by grinding the periphery of the oysters against an electric disc grinder or similar apparatus. The initial shell length (after grinding) of twenty indiscriminately selected oysters will be measured and 20 indiscriminately selected oysters will be placed in each test chamber. At the conclusion of the 96-hour test, deposition of new shell will be measured for each oyster. Since shell deposition is often asymmetrical, the length of the longest "finger" of new shell will be measured using calipers. Shell growth inhibition for each treatment group will be determined by calculating mean shell deposition as a percentage of the control. Mean shell growth inhibition in each treatment group will be expressed as a percentage of mean control shell deposition. An EC50 value will be calculated, when possible, to express the concentration of test substance that induces a 50% reduction in shell deposition.

MATERIALS AND METHODS

Test Substance

Information on the characterization of test, control or reference substances is required by Good Laboratory Practice Standards (GLP). The Sponsor is responsible for providing Wildlife International, Ltd. written verification that the test substance has been characterized according to GLPs prior to its use in the study. If written verification of GLP test substance characterization is not provided to Wildlife International, Ltd., it will be noted in the compliance statement of the final report. The attached form **IDENTIFICATION OF TEST SUBSTANCE BY SPONSOR** (Appendix I) is to be used to provide information necessary for GLP compliance.

The Sponsor is responsible for all information related to the test substance and agrees to accept any unused test substance and/or test substance containers remaining at the end of the study.

Preparation of Test Concentrations

The test substance will be administered to the test organism in water. This route of administration was selected because it represents the most likely route of exposure to aquatic organisms.

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Test Organism

The eastern oyster (*Crassostrea virginica*) will be used in this test. This species is representative of an important group of organisms and was selected for use in the test based upon past use and ease of handling in the laboratory.

Test organisms will be obtained from a commercial supplier or research facility. All organisms will be obtained from the same source and held in the laboratory for at least 10 days prior to testing. Oysters will not be used in the test if they show signs of disease or stress or if more than 5% die during the 7 days prior to the test.

Saltwater supplied to the oysters during holding and testing will be unfiltered. A saltwater alga such as *Thalassiosira sp.*, *Skeletonema sp.*, *Chaetoceros sp.* and/or *Isochrysis sp.* will be added to the water for supplemental nutrition.

Dilution Water

Water used for holding and testing will be natural unfiltered seawater collected at Indian River Inlet, Delaware. The seawater will be pumped into a 19,000-L holding tank where the salinity of the seawater will be diluted to approximately 20 ‰ (parts per thousand) with freshwater from a well on the Wildlife International, Ltd. site. The saltwater in the holding tank will be aerated by recirculation prior to delivery to the test chambers. Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents of the seawater and the results of the analyses will be summarized in the final report. Dilution water salinity should be $20 \pm 1\text{‰}$, and the pH should not vary by more than 1 unit during the test.

Test Apparatus

Test chambers will be 52-L polyethylene chambers filled with approximately 40 L of test solution. Test chambers will be impartially positioned in an environmental chamber or a temperature-controlled water bath to maintain a temperature of $22 \pm 1^{\circ}\text{C}$. An electric mixer will be placed in each test chamber to maintain the suspension of the algal diet. In addition, each test chamber will be mildly aerated to reduce the biological oxygen demand due to the feed. Test chambers will be labeled with the project number and test concentration.

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Environmental Conditions

Lighting used to illuminate the holding and test chambers will be provided by fluorescent tubes that emit wavelengths similar to natural sunlight (e.g., Colortone® 50). A photoperiod of 16 hours of light and 8 hours of dark will be controlled with an automatic timer. A 30-minute transition period of low light intensity will be provided when lights go on and off to avoid sudden changes in light intensity. Light intensity will be measured at test initiation with a SPER Scientific Ltd. light meter or equivalent.

The target test temperature will be $22 \pm 1^{\circ}\text{C}$. Temperature will be measured in each test chamber at the beginning and end of the test using a liquid-in-glass thermometer. Temperature also will be measured with a continuous recorder in the negative control chamber. Recorder measurements will be verified with a liquid-in-glass thermometer prior to test initiation.

Dissolved oxygen will be recorded in each test chamber daily. The pH will be measured in each treatment and control group at test initiation, midpoint (approximately 48 hours), and the end of the test. Dissolved oxygen will be measured using a Yellow Springs Instrument Model 51B dissolved oxygen meter, or equivalent. Measurements of pH will be made using a Fisher Accumet Model 915 pH meter, or equivalent. In the event that dissolved oxygen concentrations fall below 60% saturation, dissolved oxygen measurements will be made in every test chamber and appropriate actions will be taken after consultation with the Sponsor. Dilution water salinity will be measured in each chamber at test initiation and at the end of the test. Salinity will be measured using a Bio-Marine, Inc. AquaFauna refractometer, or equivalent. If a treatment group reaches 100% mortality, dissolved oxygen, pH and temperature measurements will be taken at that time, then discontinued.

Biological Measurements

Oysters will be inspected visually at approximately 0-24 hours and at 24, 48, 72, and 96 hours (± 1 hour) after test initiation. Oysters having open shells and not responding to gentle prodding will be considered dead and removed. Any shell abnormalities observed will be recorded. When the test is terminated, shell deposition will be recorded for each oyster by measuring the length of the longest finger of new shell. Shell growth inhibition for each treatment group will be determined by calculating mean shell deposition as a percentage of the control.

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Sampling for Analytical Measurements

Water samples will be collected from each test chamber at test initiation and every 24 hours (± 1 hour) during the test to determine concentrations of the test substance. In the event that 100% mortality occurs in any treatment, then sampling of that treatment will terminate following the next sampling interval. Samples will be collected at mid-depth from each test chamber, analyzed immediately or placed in an appropriate storage container (e.g., glass or polypropylene bottle) and stored under refrigeration ($\sim 4^{\circ}\text{C}$) until analyzed. The sample scheme is summarized below:

PROPOSED NUMBERS OF VERIFICATION SAMPLES					
Experimental Group	0 Hours	24 Hours	48 Hours	72 Hours	96 Hours
Control	1	1	1	1	1
Level 1-Low Concentration	1	1	1	1	1
Level 2	1	1	1	1	1
Level 3	1	1	1	1	1
Level 4	1	1	1	1	1
Level 5	1	1	1	1	1
	6	6	6	6	6
Total Number of Samples = 30					

The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study.

Analytical Chemistry

Chemical analysis of the samples will be performed by Wildlife International, Ltd. The analytical method used will be based upon methodology provided by the Sponsor and identified in Appendix II. The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

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Data Analysis

Mean shell growth inhibition in each treatment group will be expressed as a percentage of control growth deposition. An EC50 value will be calculated when possible to express the concentration of test substance that induced a 50% inhibition in shell deposition. The following formula will be used:

$$\% \text{Inhibition} = \frac{C - T}{C} \times 100$$

Where C = Mean growth of negative controls

T = Mean growth of treated

The data will be analyzed by linear interpolation using ICPIN computer software (when possible) (4). The no-observed-effect-concentration (NOEC) and lowest-observed-effect-concentration (LOEC) will be determined based (when possible) on a statistical analysis of the dose-response data and an assessment of the dose-response pattern.

RECORDS TO BE MAINTAINED

Records to be maintained for data generated by Wildlife International Ltd. will include, but not be limited to:

1. Copy of signed protocol.
2. Identification and characterization of the test substance, if provided by sponsor.
3. Dates of initiation and termination of the test.
4. Holding records.
5. Calculation and preparation of test concentrations.
6. Stock solution calculations and preparation, if applicable.
7. Observations.
8. If applicable, the methods used to analyze test substance concentrations and the results of analytical measurements.
9. Statistical calculations, if applicable.
10. Test conditions and physical/chemical measurements.
11. Measurements of shell deposition.
12. Copy of final report.

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FINAL REPORT

A report of the results of the study will be prepared by Wildlife International Ltd. The report will include, but not be limited to, the following, when applicable:

1. Name and address of the facility performing the study.
2. Dates upon which the study was initiated and completed. It is the responsibility of the Sponsor to provide the final date that data are recorded for chemistry, pathology and/or supporting evaluations that may be generated at other laboratories.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures as stated in the approved protocol, including any changes in the original protocol.
5. The test substance identified by name, chemical abstracts number or code number, strength, purity, and composition or other appropriate characteristics, if provided by the Sponsor.
6. Stability and the solubility of the test substances under the conditions of administration, if provided by the Sponsor or contracted to Wildlife International Ltd.
7. A description of the methods used to conduct the test.
8. A description of the test system, including the source of the test organisms, scientific name, life stage, means and ranges of lengths, observed diseases, treatments and holding procedures.
9. A description of the preparation of the test solutions, methods used to allocate organisms to test chambers and begin the test, numbers of organisms and chambers per treatment, allocation of organisms to test chambers and duration of the test.
10. A description of circumstances that may have affected the quality or integrity of the data.
11. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.
12. A description of the transformations, calculations, or operations performed on the data, a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from these analyses.
13. Statistical methods used to evaluate the data, if applicable.
14. The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.
15. The location where raw data and final report are to be stored.

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16. A statement prepared by the Quality Assurance Unit listing the dates that study inspections and audits were made and the dates of any findings reported to the Study Director and Management.
17. If it is necessary to make corrections or additions to a final report after it has been accepted, such changes will be made in the form of an amendment issued by the Study Director. The amendment will clearly identify the part of the final report that is being amended and the reasons for the amendments. Amendments will be signed and dated by the Study Director.

CHANGING OF PROTOCOL

Planned changes to the protocol will be in the form of written amendments signed by the Study Director and the Sponsor's Representative. Amendments will be considered as part of the protocol and will be attached to the final protocol. Any other changes will be in the form of written deviations signed by the Study Director and filed with the raw data. All changes to the protocol will be indicated in the final report.

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 160 and/or Part 792); OECD Principles of Good Laboratory Practice (OCDE/GD (92) 32, Environment Monograph No. 45); and Japan MAFF (59 NohSan, Notification No. 3850, Agricultural Production Bureau). Each study conducted by Wildlife International Ltd. is routinely examined by the Wildlife International Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International Ltd. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Raw data for all work performed at Wildlife International Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International Ltd. site, or at an alternative location to be specified in the final report.

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REFERENCES

- 1 U.S. Environmental Protection Agency. 1996. Series 850- Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1025: *Oyster Acute Toxicity Test (Shell Deposition)*.
- 2 U.S. Environmental Protection Agency. 1985. *Standard Evaluation Procedure, Acute Toxicity Test for Estuarine and Marine Organisms (Mollusc 96-Hour Flow-Through Shell Deposition Study)*. EPA-540/9-85-011.
- 3 ASTM Standard E729-88a. 1994. *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians*. American Society for Testing and Materials.
- 4 Norberg-King, T.J. 1993. *A Linear Interpolation Method for Sublethal Toxicity: The Inhibition Concentration (ICp) Approach*. Version 2.0. U.S. Environmental Protection Agency. National Effluent Toxicity Assessment Center. Duluth, Minnesota. Technical Report 03-93.

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APPENDIX I

IDENTIFICATION OF TEST SUBSTANCE BY SPONSOR

To be Completed by Sponsor

I. Test Substance Identity (name to be used in the report): PFOS (Perfluorooctane Sulfonic Acid Potassium Salt

Reference Standard (if applicable): Analytical Standard: N/A

Internal Standard: 1,1,2,2H,H,H,H Perfluorooctane Sulfonic Acid

Test Substance Sample Code or Batch Number: Lot 217

Test Substance Purity (% Active Ingredient): 98.9 Expiration Date: 2008

II. Test Substance Characterization

Have the identity, strength, purity and composition or other characteristics which appropriately define the test substance and reference standard been determined prior to its use in this study in accordance with GLP Standards? Yes x No

III. Test Substance Storage Conditions

Please indicate the recommended storage conditions at Wildlife International Ltd.

Ambient

Has the stability of the test substance under these storage conditions been determined in accordance with GLP Standards? Yes x No

Other pertinent stability information:

IV. Test Concentrations:

 x Adjust test concentration to 100% a.i. based upon the purity (%) given above.

 Do not adjust test concentration to 100% a.i. Test the material AS IS.

V. Toxicity Information:

Mammalian: Rat LD50 251 mg/kg Mouse LD50 N/A

Aquatic: Invertebrate Toxicity (EC/LC50) Fish Toxicity (LC50)

Daphnia magna: 27 mg/L Rainbow Trout: 11 mg/L

Daphnia magna: 50 mg/L Fathead Minnow: 38 mg/L

Other Toxicity Information (including findings of chronic and subchronic tests):

Please see MSDS

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APPENDIX II

Analytical Method Provided by Sponsor

Samples will be analyzed based upon procedures provided by the Sponsor in the following analytical methods:

1. Liquid Chromatography Mass Spectrometry (LCMS) Method for the Determination of Perfluorooctane Sulfonic Acid Potassium Salt (PFOS) In Freshwater, Saltwater and Algal Medium

A copy of the above method will be maintained in the raw data. The actual methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

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